

# Comparison of a Bioassay and a Liquid Chromatography-Fluorescence-Mass Spectrometry<sup>n</sup> Method for the Detection of Incurred Enrofloxacin Residues in Chicken Tissues<sup>1</sup>

M. J. Schneider<sup>\*,2</sup> and D. J. Donoghue<sup>†</sup>

*\*Eastern Regional Research Center, Agricultural Research Service, US Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, Pennsylvania 19038; and †University of Arkansas, Department of Poultry Science, Fayetteville, Arkansas 72701*

**ABSTRACT** Regulatory monitoring for most antibiotic residues in edible poultry tissues is often accomplished with accurate, although expensive and technically demanding, chemical analytical techniques. The purpose of this study is to determine if a simple, inexpensive bioassay could detect fluoroquinolone (FQ) residues in chicken muscle above the FDA established tolerance (300 ppb) comparable to a liquid chromatography-fluorescence-mass spectrometry<sup>n</sup> method. To produce incurred enrofloxacin (ENRO) tissues (where ENRO is incorporated into complex tissue matrices) for the method comparison, 40-d-old broilers (mixed sex) were orally dosed through drinking water for 3 d at the FDA-approved dose of ENRO (50 ppm). At the end of each day of the 3-d dosing

period and for 3 d postdosing, birds were sacrificed and breast and thigh muscle collected and analyzed. Both methods were able to detect ENRO at and below the tolerance level in the muscle, with limits of detection of 26 ppb (bioassay), 0.1 ppb for ENRO, and 0.5 ppb for the ENRO metabolite, ciprofloxacin (liquid chromatography-fluorescence-mass spectrometry<sup>n</sup>). All samples that had violative levels of antibiotic were detected by the bioassay. These results support the use of this bioassay as a screening method for examining large numbers of samples for regulatory monitoring. Positive samples should then be examined by a more extensive method, such as liquid chromatography-fluorescence-mass spectrometry<sup>n</sup>, to provide confirmation of the analyte.

(Key words: bioassay, broiler tissue, enrofloxacin, fluoroquinolone, liquid chromatography-fluorescence-mass spectrometry<sup>n</sup>)

2004 Poultry Science 83:830–834

## INTRODUCTION

Fluoroquinolone (FQ) antibiotics display a wide range of antibacterial activity and are used in medical and veterinary applications. The use of antibiotics in food animals has generated concern due to increased reports of antibiotic resistance (World Health Organization, 1998) and consumer concerns about the safety of antibiotic residues in foods (Resurreccion and Galvez, 1999; Donoghue 2003). Enrofloxacin (ENRO) is currently the only FQ approved for use in broiler chickens in the US, although additional FQ are used worldwide (World Health Organization, 1998). The US FDA has set a tolerance level of 300 ppb ENRO in broiler muscle, and a 2-d withdrawal period to allow time for the drug concentration to decrease to an

acceptable level prior to slaughter, thus minimizing the possibility of violative residues (Code of Federal Regulations, 2003). It is also important to monitor ENRO in broiler muscle to ensure the absence of samples containing ENRO above the tolerance level in order to promote consumer confidence in the safety of the food supply.

A full and complete analysis of broiler muscle would provide the actual concentration (if any) of the analyte present (quantitation) and would verify the identity of the analyte as ENRO (confirmation). There are a number of methods that have been used to analyze ENRO in chicken muscle (Waggoner and Bowman, 1987; Horie et al., 1994; Rose et al., 1998; Yorke and Froc, 2000; Barron et al., 2001; Garcia et al., 2001; Horstkotter et al., 2002; Schneider and Donoghue, 2002), although not all of these would provide both quantitation and confirmation. We have developed an efficient method using liquid chromatography, fluorescence, and mass spectrometry<sup>n</sup> (LC-FL-

©2004 Poultry Science Association, Inc.

Received for publication October 17, 2003.

Accepted for publication December 19, 2003.

<sup>1</sup>Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

<sup>2</sup>To whom correspondence should be addressed: mschneider@errc.ars.usda.gov.

**Abbreviation Key:** CIP = ciprofloxacin; ENRO = enrofloxacin; FQ = fluoroquinolone; LC-FL-MS<sup>n</sup> = liquid chromatography-fluorescence-mass spectrometry<sup>n</sup>.

MS<sup>n</sup>), which allows for simultaneous quantitation and confirmation of 8 FQ, including ENRO, in chicken muscle (Schneider and Donoghue, 2002). Potential drawbacks of these methods include the need for fairly expensive instrumentation and trained personnel to run them. In addition, the sample preparation and clean-up steps may increase difficulty of the application to very large numbers of samples.

A large number of broiler chickens are produced annually in the US. For example, more than 8 billion broilers were produced in the US in 2001 ([www.usda.gov/nass/aggraphs/brlprod.htm](http://www.usda.gov/nass/aggraphs/brlprod.htm) Accessed Sept. 2003). Although not every chicken need be analyzed, the ability to quickly and inexpensively analyze large numbers of samples is an advantage. The latest regulatory monitoring of antibiotics in poultry tissues indicates a less than 1% incidence of violations from 1997 to 2000 ([www.fsis.usda.gov/OPHS/red2000/index.htm](http://www.fsis.usda.gov/OPHS/red2000/index.htm)). The ability to process large numbers of samples to detect these few violative tissues would be facilitated with the use of a screening method as proposed in this study. The few positive samples could then be re-analyzed by a more rigorous analytical method to provide the desired quantitation and confirmation information. Identification of violative samples would allow regulatory intervention to correct potential misuse of these antibiotics and to ensure consumer confidence in these poultry products.

We have developed a simple and rapid bioassay method for the analysis of ENRO in biological samples. The bioassay allows facile analysis of a large number of samples ( $n = 120/2$  days), relative to the LC-FL-MS<sup>n</sup> method ( $n = 12/\text{day}$ ), and recently was successfully tested against the LC-FL-MS<sup>n</sup> method for analysis of ENRO in incurred eggs (Donoghue and Schneider, 2003). In this report, we compare the bioassay method with the LC-FL-MS<sup>n</sup> method for analysis of incurred ENRO in broiler muscle. Breast and thigh muscle samples were examined to determine if these methods produce comparable results for these different muscle tissues.

## MATERIALS AND METHODS

### *Incurred and Control Tissues*

Incurred muscle samples were generated by dosing 16 broilers (40 d of age, mixed sex) with the FDA-approved concentration of 50 ppm ENRO in the water for 3 d at the University of Arkansas poultry farm. Incurred muscle samples were evaluated because they exemplify how antibiotics are actually incorporated into the complex muscle matrices and are representative of food consumed by the public and collected for regulatory monitoring. As per label directions, the medicated water was made fresh daily. At the end of d 1, 2, 3, and for up to 3 d after withdrawal of ENRO, 2 birds were killed daily, and the

entire breast and thigh muscles were collected. During the experiment, birds had ad libitum access to a standard, nonmedicated broiler diet (19.5% CP, 3,150 kcal of ME/kg, 0.9% Ca and 0.35% available P) and water. After collection, the entire breast or thigh muscle tissues from the 2 birds were combined and homogenized. One portion of these homogenized tissues was stored at the University of Arkansas for analysis by bioassay, and another portion was shipped overnight on dry ice to the USDA facilities in Pennsylvania for analysis by LC-FL-MS<sup>n</sup>. All samples were stored at  $-80^{\circ}\text{C}$  until analysis. Control muscle was either collected from undosed broilers or purchased<sup>3</sup> as "antibiotic free" from a local store and analyzed to confirm that FQ were absent.

### *Bioassay Procedure*

Samples were assayed using an agar diffusion microbiological method adapted from Donoghue and Schneider (2003). After thawing, incurred muscle samples were diluted 1:3 (vol/vol) with 1% phosphate buffer, pH 9.0 and centrifuged at  $1,500 \times g$  for 20 min at  $5^{\circ}\text{C}$ ; the supernatant was decanted and used for analysis. Either a matrix matched (muscle) or buffer standard curve was constructed by addition of known concentrations of ENRO to the muscle or buffer. The matrix matched samples were diluted with buffer, centrifuged, and decanted as described above for the incurred samples. The presence of matrix (muscle) did not interfere with the analysis, as results indicated no differences between the matrix matched or buffer standard curve (data not shown). Petri dishes (100 mm in diameter) were filled with 8 mL of Muller Hinton agar, and 6 penicylinders<sup>3</sup> ( $8 \times 10$  mm) were evenly placed on the agar. Prior to filling, the agar was inoculated with approximately  $1 \times 10^6$  cfu/mL *Klebsiella pneumoniae* (ATCC 10031). Each standard concentration was pipetted onto 3 plates; 3 alternate cylinders were filled with standard (200  $\mu\text{L}$ ), and the other 3 cylinders were filled (200  $\mu\text{L}$ ) with a reference concentration. Individual incurred samples were assayed in a similar manner except samples were assayed on only 1 plate. The plates were incubated for approximately 16 h at  $37^{\circ}\text{C}$ . Plate averages for the standards and incurred samples were corrected to the overall reference concentration. A best-fit regression line using the diameter of the growth inhibition zones (millimeters: Zone Reader<sup>4</sup>) was calculated by the method of least squares.

### *LC-FL-MS<sup>n</sup> Procedure*

Muscle samples (1.0 g) were extracted with acetonitrile/ammonium hydroxide and processed following the procedure described previously (Donoghue and Schneider, 2003), except that all centrifugations were performed at  $2,791 \times g$ . Incurred samples from d 1 to 4 were diluted with control muscle to provide samples for analysis within range of the standard curve ( $\leq 100$  ppb FQ). Samples from d 5 and 6 were analyzed without dilution.

<sup>3</sup>Bell and Evans, Fredericksburg, PA.

<sup>4</sup>Fisher, Pittsburgh, PA.

Analyses were performed as described previously (Donoghue and Schneider, 2003), using a Hewlett-Packard 1100 series liquid chromatograph binary pump with on-line degasser, autosampler, column heater, fluorescence detector and Eclipse XDB-phenyl  $3.0 \times 150$  mm,  $3.5 \mu\text{m}$  HPLC column<sup>5</sup> with inline  $2\text{-}\mu\text{m}$  filter and  $\text{C}_{18}$ ,  $2.0$  mm i.d. Security Guard column cartridge.<sup>6</sup> Eluent from the fluorescence detector passed directly into the LCQ-DECA mass spectrometer.<sup>7</sup> XCalibur software version 1.2<sup>7</sup> controlled the liquid chromatography and mass spectrometer systems and processed data from the fluorescence detector with a SS-420X module.<sup>8</sup> An external standard curve for 8 FQ, including ENRO and ciprofloxacin (CIP), was generated daily and fluorescence peak height was used for quantitation. Confirmation was achieved by comparison of the ratios of 2 major  $\text{MS}^2$  (desethyle CIP) or  $\text{MS}^3$  (norfloxacin, CIP, danofloxacin, ENRO, orbifloxacin, sarafloxacin, difloxacin) product ions with those of standard samples.

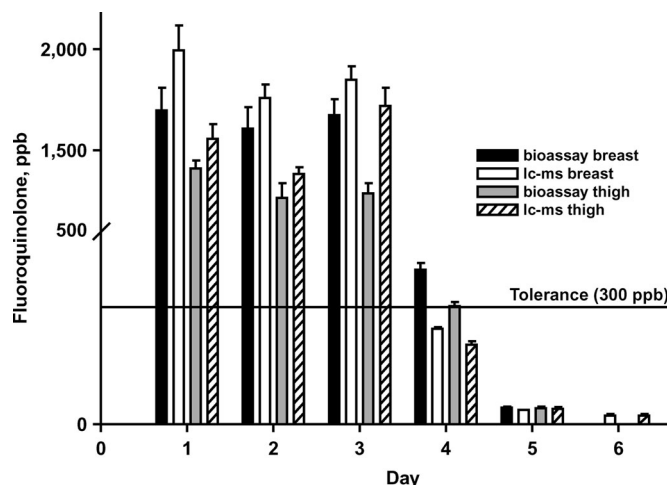
### Statistical Analysis

Data were analyzed by ANOVA using the Statistical Analysis System (SAS, 1994) general linear models program. Treatment means were partitioned by least squares means analysis (SAS, 1994). A probability of  $P < 0.05$  was required for statistical significance.

## RESULTS AND DISCUSSION

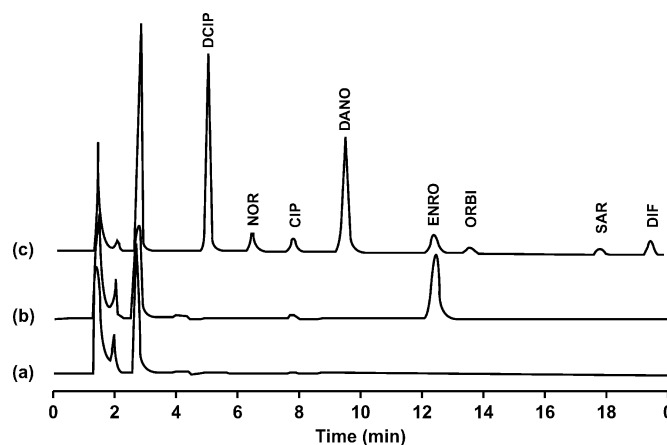
Breast and thigh muscle samples from d 1 to 3 of dosing and d 4 to 6 (postdose) were analyzed by the bioassay and the LC-FL- $\text{MS}^n$  methods for FQ concentration, and the results are shown in Figure 1. During the 3-d dosing period, FQ concentrations greatly exceeded the 300 ppb tolerance established by the FDA for poultry tissues, on d 4, FQ residues were close to the 300 ppb tolerance, and on d 5 and 6, the levels were below tolerance. Both methods detected the violative residue levels in breast and thigh muscles during the 3-d dosing period and non-violative concentrations on d 5 and 6. On d 4, data obtained from each assay were close to the ENRO tolerance level of 300 ppb. The bioassay indicated the breast and thigh samples to be violative (396 and 303 ppb, respectively), whereas the LC-FL- $\text{MS}^n$  method gave nonviolative results (246 and 205 ppb, respectively). The bioassay results showed that it could be used to detect all samples close to or above the ENRO tolerance established by the FDA. These data support its use as an effective screening method and also lend support to the FDA's decision to require a 2-d withdrawal period to allow residue concentrations to deplete below the established tolerance.

Although the bioassay may be a useful method for screening large numbers of samples for ENRO, the ENRO



**FIGURE 1.** Bioassay or liquid chromatography-fluorescence-mass spectrometry<sup>n</sup> quantitation of fluoroquinolone concentrations (ppb) detected in the same incurred breast or thigh samples (mean  $\pm$  SD). Incurred samples were collected from chickens following oral dosing with enrofloxacin (ENRO 50 ppm) for 3 d. ENRO concentrations detected were different between the 2 assays within days, except for d 2 ( $P > 0.05$ ). Sample size:  $n = 6$ /day per tissue type.

concentrations detected were different between the 2 assays except for d 2 ( $P > 0.05$ ). Our previous study comparing these 2 methods for the detection of ENRO in eggs demonstrated comparable quantitation (Donoghue and Schneider, 2003). The differences between these 2 studies may be due to an increased difficulty of extraction of FQ from muscle as opposed to eggs. The bioassay uses an easier, although less extensive, extraction method (vortex mixing in buffer) than the LC-FL- $\text{MS}^n$  method (homogenization in solvent). Our use of incurred tissues (e.g., when chickens are dosed with ENRO, and the ENRO is incorporated into complex tissue matrices) enhances our ability to detect differences between these assays due to sample preparation and matrix interference associated with dif-



**FIGURE 2.** Liquid chromatograms of (a) extract of control thigh; (b) extract of enrofloxacin (ENRO)-incurred thigh, d 4; (c) extract of control thigh fortified with 100 ppb desethyle ciprofloxacin (DCIP), norfloxacin (NOR), ciprofloxacin (CIP), danofloxacin (DANO), ENRO, orbifloxacin (ORBI), sarafloxacin (SAR), and difloxacin (DIF). The Y-axis represents fluorescence intensity.

<sup>5</sup>Agilent, Wilmington, DE.

<sup>6</sup>Phenomenex, Torrance, CA.

<sup>7</sup>ThermoQuest, San Jose, CA.

<sup>8</sup>Scientific Software, Pleasanton, CA.

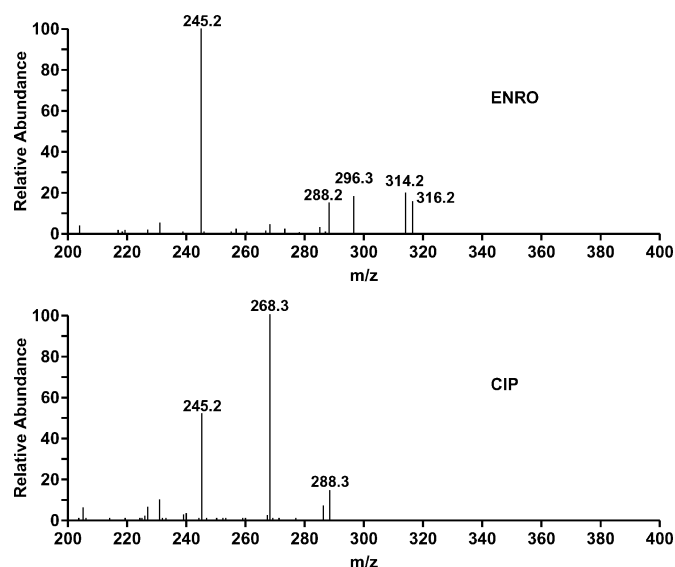


FIGURE 3. Representative mass spectrometry ( $MS^3$ ) spectra of ciprofloxacin (CIP) and enrofloxacin (ENRO) in ENRO-incurred thigh, d 4. Major product ion ratios ( $m/z$  288/245 for ENRO,  $m/z$  245/268 for CIP) can be compared with those of standard samples to provide confirmation.

ferent tissues. The use of fortified samples (e.g., when ENRO is added on top of the control egg or muscle tissues during sample preparation) reduces the potential for these types of issues. Although fortified samples may thus be easier to work with, they are not the preferred option for method evaluation. Incurred samples are a better choice, as these samples are representative of the actual food sample collected for monitoring and consumed by the public. Therefore, our use of incurred samples gives greater confidence in the ability of the bioassay to detect violative samples than an assay developed using only fortified samples. Another difference between these assays is the inability of the bioassay to identify different

FQ moieties. Lack of confirmatory ability is a common problem for bioassays (Calderon et al., 1996; Braham et al., 2001; Dey et al., 2003). Our bioassay was not able to differentiate between ENRO residues and the ENRO metabolite ciprofloxacin (CIP). Using the LC-FL- $MS^n$  method, CIP was detected at concentrations ranging from  $1.72 \pm 0.35$  ppb (d 6) to  $72.2 \pm 16.3$  ppb (d 3) in thigh and from  $8.58 \pm 0.41$  ppb (d 4) to  $75.9 \pm 7.4$  ppb (d 3) in breast. The lack of confirmatory ability does not hinder the ability of the bioassay to detect violative or nonviolative FQ residues (Figure 1). As stated previously, follow-up testing will be required to demonstrate the existence of specific FQ present in violative samples detected by this screening procedure.

In this study, samples of breast and thigh muscle were used to determine any potential differences between the assays due to the differences in these edible broiler tissues. Both assays performed comparably in their abilities to detect either violative or nonviolative residues in these tissues. It is interesting to note, however, that breast muscle appeared to have consistently higher FQ concentrations than thigh muscle, as measured by both assays (Figure 1). Although this study was not designed to address differences in the kinetics of ENRO deposition in these tissues, follow-up studies are planned to evaluate this possible effect. If differences are detected, it would be important for regulatory monitoring of poultry to test the muscle tissue with the highest potential residue concentrations to ensure the safety of the product. Differences in residue deposition within muscle tissues have been observed previously for other antibiotics (Beechinor and Bloomfield, 2001).

Whereas the bioassay has the advantages of simplicity and lower cost, the more extensive LC-FL- $MS^n$  method is capable of providing excellent quantitation through fluorescence and confirmation of the FQ through  $MS^n$ . Chromatograms in Figure 2 clearly show the absence of

TABLE 1. Mass spectrometry confirmation data of ENRO<sup>1</sup> and CIP<sup>2</sup> residues in incurred broiler tissues

Sample	CIP peak ratio (% 245/268)	n	Sample	ENRO peak ratio (% 288/245)	n
Breast			Breast		
Standard FQ <sup>3</sup> mix 1-100 ppb	47.6 $\pm$ 5.4	88	Standard FQ mix 5-100 ppb	16.7 $\pm$ 2.3	66
Day 1 incurred	45.8 $\pm$ 5.5	12	Day 1 incurred	16.6 $\pm$ 1.7	12
Day 2 incurred	50.1 $\pm$ 5.9	12	Day 2 incurred	16.4 $\pm$ 1.9	12
Day 3 incurred	45.5 $\pm$ 5.9	12	Day 3 incurred	16.1 $\pm$ 1.1	12
Day 4 incurred	45.3 $\pm$ 4.7	12	Day 4 incurred	17.0 $\pm$ 1.8	12
Day 5 incurred	47.2 $\pm$ 9.8	12	Day 5 incurred	16.8 $\pm$ 1.0	12
Day 6 incurred	43.1 $\pm$ 17.4	12	Day 6 incurred	15.0 $\pm$ 3.8	12
Thigh			Thigh		
Standard FQ mix 1-100 ppb	46.5 $\pm$ 4.9	86	Standard FQ mix 5-100 ppb	16.3 $\pm$ 2.3	62
Day 1 incurred	47.6 $\pm$ 7.5	12	Day 1 incurred	16.7 $\pm$ 1.3	12
Day 2 incurred	48.6 $\pm$ 7.6	12	Day 2 incurred	16.4 $\pm$ 1.3	12
Day 3 incurred	50.0 $\pm$ 4.4	12	Day 3 incurred	16.5 $\pm$ 1.5	12
Day 4 incurred	47.9 $\pm$ 6.1	12	Day 4 incurred	16.1 $\pm$ 1.2	12
Day 5 incurred	49.0 $\pm$ 10.0	12	Day 5 incurred	16.9 $\pm$ 3.4	12
Day 6 incurred	44.2 $\pm$ 9.2	12	Day 6 incurred	16.5 $\pm$ 2.3	12

<sup>1</sup>ENRO = enrofloxacin.

<sup>2</sup>CIP = ciprofloxacin.

<sup>3</sup>FQ = fluoroquinolones.

FQ in control muscle tissue and the presence of ENRO in a representative incurred sample. Representative MS<sup>3</sup> spectra of ENRO and CIP are displayed in Figure 3, illustrating the product ions used for confirmation. Confirmation of the FQ is achieved by comparison of major product ion ratios ( $m/z$  288/245 for ENRO and  $m/z$  245/268 for CIP) in unknown samples with those from standard samples. Confirmation data for the samples analyzed in this study are listed in Table 1. The comparable values for these ratios between the incurred and standard samples provides strong evidence for the presence of CIP and ENRO in the incurred samples.

In conclusion, this study has found the bioassay to be a very useful tool for initial screening of a large number of samples for the presence of FQ in ENRO-incurred chicken muscle. The bioassay's low limit of detection relative to tolerance and its ability to identify violative samples, coupled with its ease of use, should make it a valuable addition to the regulatory methods available. The relatively few apparently violative samples identified by such a screening method would then be subjected to analysis by a more involved method such as LC-FL-MS<sup>n</sup>, which could then provide confirmation as well as quantitation. Use of both methods in this manner would then provide a highly efficient approach to analysis of large numbers of samples for FQ residues.

## ACKNOWLEDGMENTS

We thank Susan Braden and Pam Blore for technical assistance; Bayer for providing standard samples of enrofloxacin, desethyle ciprofloxacin, and ciprofloxacin; Pfizer for a sample of danofloxacin; Abbott Laboratories for samples of sarafloxacin, and difloxacin and Schering Plough for a standard of orbifloxacin.

## REFERENCES

- Barron, D., E. Jimenez-Lozano, J. Cano, and J. Barbosa. 2001. Determination of residues of enrofloxacin and its metabolite ciprofloxacin in biological materials by capillary electrophoresis. *J. Chromatogr. B. Biomed. Sci. Appl.* 759:73–79.
- Beechinor, J. G., and F. J. Bloomfield. 2001. Variability in residue concentrations of tilmicosin in cattle muscle. *Vet. Rec.* 149:182–183.
- Braham, R., W. D. Black, J. Claxton, and A. J. Yee. 2001. A rapid assay for detecting sulfonamides in tissues of slaughtered animals. *J. Food Prot.* 64:1565–1573.
- Calderon, V., J. Gonzalez, P. Diez, and J. A. Berenquer. 1996. Evaluation of a multiple bioassay technique for determination of antibiotic residues in meat with standard solutions of antimicrobials. *Food Addit. Contam.* 13:13–19.
- Code of Federal Regulations. 2003. Enrofloxacin oral solution. 21CFR520.813; and Enrofloxacin 21CFR556.228. [www.gpoaccess.gov/cfr/index.html](http://www.gpoaccess.gov/cfr/index.html). Accessed Oct. 2003.
- Dey, B. P., A. Thaler, and F. Gwozdz. 2003. Analysis of microbiological screen test data for antimicrobial residues in food animals. *J. Environ. Sci. Health B* 38:391–404.
- Donoghue, D. J. 2003. Antibiotic residues in poultry tissues and eggs: Human health concerns? *Poult. Sci.* 82:618–621.
- Donoghue, D. J., and M. J. Schneider. 2003. Comparison between a bioassay and liquid chromatography-fluorescence-mass spectrometry<sup>n</sup> for the determination of incurred enrofloxacin in whole eggs. *J. AOAC Int.* 86:669–674.
- Garcia, M. A., C. Solans, E. Hernandez, M. Puig, and M. A. Bregante. 2001. Simultaneous determination of enrofloxacin and its primary metabolite, ciprofloxacin, in chicken tissues. *Chromatographia* 54:191–194.
- Horie, M., K. Saito, N. Nose, and H. Nakazawa. 1994. Simultaneous determination of benofloxacin, danofloxacin, enrofloxacin and ofloxacin in chicken tissues by high performance liquid chromatography. *J. Chromatogr. B. Biomed. Appl.* 653:69–76.
- Horstkotter, C., E. Jimenez-Lozano, D. Barron, J. Barbosa, and G. Blaschke. 2002. Determination of residues of enrofloxacin and its metabolite ciprofloxacin in chicken muscle by capillary electrophoresis using laser-induced fluorescence detection. *Electrophoresis* 23:3078–3083.
- Resurreccion, A. V. A., and F. C. F. Galvez. 1999. Will consumers buy irradiated beef? *Food Technol.* 53:52–55.
- Rose, M. D., J. Bygrave, and G. W. F. Stubbings. 1998. Extension of multi-residue methodology to include the determination of quinolones in food. *Analyst* 123:2789–2796.
- SAS Institute. 1994. SAS User's Guide. 4th ed. SAS Institute, Cary, NC.
- Schneider, M. J., and D. J. Donoghue. 2002. Multiresidue analysis of fluoroquinolone antibiotics in chicken tissue using liquid chromatography-fluorescence-multiple mass spectrometry. *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.* 780:83–92.
- Waggoner, T. B., and M. C. Bowman. 1987. Spectrofluorometric determination of BAY Vp 2674 residues in poultry tissues. *J. AOAC Int.* 70:813–818.
- World Health Organization. 1998. Use of quinolones in food animals and potential impact on human health. WHO, Geneva, Switzerland.
- Yorke, J. C., and P. Froc. 2000. Quantitation of nine quinolones in chicken tissues by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A* 882:63–77.